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PRENATAL ADMINISTRATION OF OLEIC ACID OR LINOLENIC ACID REDUCES NEUROMORPHOLOGICAL AND COGNITIVE ALTERATIONS IN TS65DN DOWN SYNDROME MICE

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Corresponding Author:	Carmen Martínez-Cué, Ph.D. Universidad de Cantabria Facultad de Medicina Santander, SPAIN
Corresponding Author's Institution:	Universidad de Cantabria Facultad de Medicina
First Author:	Susana García-Cerro
Order of Authors:	Susana García-Cerro
	Noemí Rueda
	Verónica Vidal
	Alba Puente
	Victor Campa
	Sara Lantigua
	Oriol Narcís
	Ana Velasco
	Renata Bartesaghi
	Carmen Martínez-Cué, Ph.D.
Abstract:	Background: The cognitive impairments that characterize Down syndrome (DS) have been attributed to brain hypocellularity due to neurogenesis impairment during fetal stages. Thus, enhancing prenatal neurogenesis in DS could prevent or reduce some of the neuromorphological and cognitive defects found in post-natal stages.Objectives: Because fatty acids play a fundamental role in morphogenesis and brain development during fetal stages, in this study, we aimed to enhance neurogenesis and the cognitive abilities of the Ts65Dn (TS) mouse model of DS by administering oleic or linolenic acid.Methods: Eighty-five pregnant TS females were subcutaneously treated from embryonic day (ED) 10 until postnatal day (PD) 2 with oleic acid (400 mg/kg), linolenic acid (500 mg/kg), or vehicle. All analyses were performed on their TS and Control (CO) male and female progeny. At PD2, we evaluated the short-term effects of the treatments on neurogenesis, cellularity, and brain weight, in 40 TS and CO pups. Sixty-nine TS and CO mice were used to test the long-term effects of the prenatal treatments on cognition from PD30 to PD45, and on neurogenesis, cellularity, and synaptic markers, at PD45. Data were compared by ANOVAs.Results: Prenatal administration of oleic or linolenic acid increased the brain weight (+36.7% and +45%, P<0.01), the density of BrdU- (Bromodeoxyuridine) (+80% and +115%; P<0.01), and DAPI (4′,6-diamidino-2-phenylindole)-positive cells (+64% and +22%, P<0.05) of PD2 TS mice with respect to the vehicle-treated TS mice. Between PD30 and PD45, TS mice prenatally-treated with oleic or linolenic acid showed better cognitive abilities (+28% and +25%, P<0.01) and a higher density of the post-synaptic marker PSD95

	(Postsynaptic Density Protein 95) (+65% and +44%, P<0.05) than the vehicle-treated TS animals.Conclusion: The beneficial cognitive and neuromorphological effects induced by oleic or linolenic acid in TS mice suggest that they could be promising pharmacotherapies for DS-associated cognitive deficits.
Response to Reviewers:	Comments from the Editors and Reviewers: Assistant Editor 18-25: Please add quantitative data or the magnitude of differences for key variables. Response: We have now included the magnitude of the changes after oleic or linolenic acid administration for key variables. Please check the statistical test results shown in fig 1A and supplemental fig 1 B,C, E, and F. The greatest and least values should carry a single letter. e.g. a mean labeled 'ab' should not be greater than a mean labeled 'a'. Means labeled abc or bc should not be greater than means labeled b. Please verify that all statistical test results are correct as shown in all tables and figures. Response: We have corrected the statistical results shown in figure 1A and supplemental figures 1B, C, E and F. We have also revised the rest of the figures and we believe that they are now correctly shown in all of them. Verify that at < 18 cm width, all reduced text on figures (especially figure 4) is 6-8 points. If it is smaller, it will not be legible in print. Response: We have increased the size of some of the fonts in figures 4 and 5, and verified for the rest of the figures that at <18 cm all letters are legible.
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PRENATAL ADMINISTRATION OF OLEIC ACID OR LINOLENIC ACID REDUCES NEUROMORPHOLOGICAL AND COGNITIVE ALTERATIONS IN TS65DN DOWN SYNDROME MICE

García-Cerro, Susana[1*]; Rueda, Noemí[1*]; Vidal, Verónica[1]; Puente, Alba[1]; Campa, Víctor[2]; Lantigua, Sara[1]; Narcís, Oriol[1]; Velasco, Ana[3]; Bartesaghi, Renata[4]; Martínez-Cué, Carmen[1#]

[1] Department of Physiology and Pharmacology, Faculty of Medicine, University of Cantabria, Santander, Spain

[2] Institute of Molecular Biology and Biomedicine (IBTECC), Santander, Cantabria, Spain.

[3] Department of Biochemistry and Molecular Biology. Institute of Neurosciences of Castilla and Leon (INCYL). University of Salamanca. Institute of Biomedical Research of Salamanca (IBSAL), Spain

[4] Department of Biomedical and Neuromotor Sciences, University of Bologna, Bologna, Italy

*: These authors contributed equally to this work

[#]: Corresponding author (Carmen Martínez-Cué) contact information:

Mailing address: Department of Physiology and Pharmacology, Faculty of Medicine, c/ Cardenal Herrera Oria s/n, Santander, 39011, Spain.

Telephone number: (+34) 942203935

Fax: (+34) 942201903

email: martinec@unican.es

List of all authors' last names exactly as they should appear for PubMed indexing: Garcia-Cerro; Rueda; Vidal, Puente; Campa; Lantigua; Narcis; Velasco; Bartesaghi; Martinez-Cue

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i. **Supplemental figure 1** is available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents.

ii. Abbreviations: AFP: alpha-fetoprotein; AD: Alzheimer’s disease; ANOVA: Analysis of Variance; ARA: arachidonic acid; BrdU: Bromodeoxyuridine; BSA: (Bovine Serum Albumin); CO: Control; CO-LNA: Control linolenic acid; CO-OA: Control oleic acid; CO-V: Control vehicle; DAPI: 4′,6-diamidino-2-phenylindole; DG: Dentate gyrus; DHA: Docosahexaenoic acid; DS: Down syndrome; ED: Embryonic Day; GCL: Granular cell layer; HPLC: High-Performance Liquid Chromatography; LSD: Least significant difference; LTP: Long-term potentiation; ML: Molecular layer; MWM: Morris water maze; NPC: Neural progenitor cells; PB: Phosphate buffer; PBS: Phosphate-buffered saline; PD: Postnatal Day; PFA: Paraformaldehyde; PSD95: Postsynaptic density protein 95; PUFA: Polyunsaturated fatty acid; qPCR: Quantitative polymerase chain reaction; RM: Repeated measures; SGZ: Subgranular zone; SYN: synaptophysin; TS:

Ts65Dn; TS-LNA: TS linolenic acid; TS-OA: TS oleic acid; TS-LV: TS-vehicle; TX: Triton X; VGluT: Vesicular glutamate transporter.

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iv. Conflict of interests: All the authors declare no conflict of interest.

ABSTRACT

Background: The cognitive impairments that characterize Down syndrome (DS) have been attributed to brain hypocellularity due to neurogenesis impairment during fetal stages. Thus, enhancing prenatal neurogenesis in DS could prevent or reduce some of the neuromorphological and cognitive defects found in post-natal stages.

Objectives: Because fatty acids play a fundamental role in morphogenesis and brain development during fetal stages, in this study, we aimed to enhance neurogenesis and the cognitive abilities of the Ts65Dn (TS) mouse model of DS by administering oleic or linolenic acid.

Methods: Eighty-five pregnant TS females were subcutaneously treated from embryonic day (ED) 10 until postnatal day (PD) 2 with oleic acid (400 mg/kg), linolenic acid (500 mg/kg), or vehicle. All analyses were performed on their TS and Control (CO) male and female progeny. At PD2, we evaluated the short-term effects of the treatments on neurogenesis, cellularity, and brain weight, in 40 TS and CO pups. Sixty-nine TS and CO mice were used to test the long-term effects of the prenatal treatments on cognition from PD30 to PD45, and on neurogenesis, cellularity, and synaptic markers, at PD45. Data were compared by ANOVAs.

Results: Prenatal administration of oleic or linolenic acid increased the brain weight (+36.7% and +45%, $P<0.01$), the density of BrdU- (Bromodeoxyuridine) (+80% and +115%; $P<0.01$), and DAPI (4′,6-diamidino-2-phenylindole)-positive cells (+64% and +22%, $P<0.05$) of PD2 TS mice with respect to the vehicle-treated TS mice. Between PD30 and PD45, TS mice prenatally-treated with oleic or linolenic acid showed better cognitive abilities (+28% and +25%, $P<0.01$) and a higher density of the post-synaptic marker PSD95 (Postsynaptic Density Protein 95) (+65% and +44%, $P<0.05$) than the vehicle-treated TS animals.

Conclusion: The beneficial cognitive and neuromorphological effects induced by oleic or linolenic acid in TS mice suggest that they could be promising pharmacotherapies for DS-associated cognitive deficits.

Keywords: Down syndrome; Ts65Dn mice; oleic acid; linolenic acid; prenatal treatment; neurogenesis; cognition.

LAY SUMMARY

Down syndrome (DS) is characterized by cognitive dysfunction due to alterations in neurogenesis during prenatal stages. Because linolenic and oleic acid increase neurogenesis in these stages, its administration could improve the cognitive abilities of DS individuals. We aimed to test whether prenatal administration of these acids could restore the brain alterations (i.e. neurogenesis and cellularity) and the cognitive impairments in the Ts65Dn (TS) mouse model of DS. We treated pregnant TS females with oleic acid, linolenic acid or vehicle and we analyzed neurogenesis and cellularity in their newborn pups. TS pups that received prenatally oleic or linolenic acid presented higher neurogenesis and cellularity than those that received vehicle. To determine whether these prenatal treatments had long-term effects on cognition, neurogenesis and the number of synapses, another group of mice whose mothers received oleic acid, linolenic acid or vehicle during gestation was used. At the age of 4-6 weeks, TS mice that received prenatally oleic or linolenic acid presented better cognitive abilities and a higher density of synapses than TS mice treated prenatally with vehicle. The beneficial effects observed in TS mice suggest that oleic acid and linolenic acid could be promising therapies to treat the cognitive disabilities of DS.

INTRODUCTION

Down syndrome (DS), the most common genetic cause of intellectual disability, is characterized by numerous neurobiological alterations. One of the factors partially responsible for the cognitive impairments in DS is the brain hypocellularity due to alterations in neurogenesis during the early developmental stages (1-4).

The Ts65Dn (TS) mouse, the most commonly used DS model, resembles many of its phenotypes exhibiting altered cognitive abilities, hypocellularity, and reduced neurogenesis (4, 5-7). Thus, therapies aimed at rescuing neurogenesis might be a good approach to treating intellectual disability in DS individuals. Several pharmacotherapies have been reported to rescue the neurogenesis and cognitive deficits in DS murine models when administered in the pre- or post-natal stages (8-20). However, some of these drugs have failed to produce any benefit in humans or cannot be safely administered to individuals with DS. In addition, DS individuals and TS mice present altered development of the brain, beginning at the fetal stages (2, 3, 21). Therefore, these alterations should be corrected from the early developmental stages, using safe drugs or natural substances that can be administered during the prenatal or early postnatal stages (2, 10, 11, 22). In this context, the administration of fatty acids could be a promising strategy.

There is a direct association between the percentage of fatty acids in maternal plasma and the development of cognitive function in neonates (23), and exogenous administration of fatty acids during the pre- and post-natal periods increases brain development in humans and other animals (24). Oleic acid is a monounsaturated acid of the omega-9 series. It is present endogenously in the organism and it can also be obtained from the diet. Oleic acid acts as a neurotrophic factor in initial life stages, inducing neuronal differentiation, the growth of new neurites, neuronal migration, and synapse formation (25-30).

α-Linolenic acid is an essential Polyunsaturated Fatty Acid (PUFA) of the omega-3 series, which has to be obtained from the diet. Linolenic acid is essential for proper brain development; its deficit can affect neurogenesis and neuronal function (31-33). In rodents, supplementation of linolenic acid or its derivative Docosahexaenoic Acid (DHA) in the diet of pregnant females enhances cognition and neurogenesis in the brains of their offspring (32-51).

In DS, the lower levels of fatty acids in brain phospholipids (52), could be partially responsible for some of the neuromorphological and cognitive alterations encountered in this syndrome. Thus, treatment with fatty acids during the critical windows of neurogenesis (the prenatal and early postnatal periods) may induce beneficial effects. In addition, oleic acid and linolenic acid do not produce important side effects, and their efficacy in treating different pathologies is currently being evaluated (53-55). Thus, the aim of this study was to analyze whether prenatal administration of oleic or linolenic acid restores the neuromorphological alterations found in TS mice, and whether these effects are maintained after discontinuation of the treatment, leading to enhanced cognitive abilities.

METHODS

Animals, Diets and Treatments

This study was approved by the Cantabria University Institutional Laboratory Animal Care and Use Committee and performed in accordance with the Declaration of Helsinki and the European Communities Council Directive (86/609/EEC).

TS mice were generated by repeated backcrossing of B6EiC3Sn a/A-Ts(17<16>)65Dn females with C57BL/6Ei × C3H/HeSNJ (B6EiCSn) F1 hybrid males. TS mice were compared with euploid littermates (Control, CO). Trisomy was determined by real-time qPCR (quantitative Polymerase Chain Reaction) as previously described (56).

Diet

All pregnant and lactating TS mice were fed with Tekcal 18% protein (#2018, containing 18.6% raw protein, 6.2% fat and 44.2% carbohydrates) Global Mouse Chow, specially formulated for gestation and lactation (INVIGO, Huntingdon, UK), from ED (Embryonic Day) 0 until the weaning of the pups. For the study of the long-term effects, all TS and CO mice received Tekcal Mouse Chow 14% protein (#2014, INVIGO, containing 14.3 % raw protein, 4.0% fat and 48.0% carbohydrates), designed to promote normal body weight and longevity in the rodents from weaning (Postnatal Day (PD) 21) to the end of the study.

Treatments

A total of eighty-five pregnant TS females were subcutaneously treated with oleic acid (400 mg/kg), linolenic acid (500 mg/kg), or vehicle (Bovine Serum Albumin (BSA) 10 %) from ED10 until PD2). The doses selected are neuroprotective and/or induce neurogenesis (35, 47, 57, 58). Plasma concentrations of the three compounds in the PD2 pups were quantified by High-Performance Liquid Chromatography (HPLC). Male

and female TS and CO pups gestated by forty-five TS females under the different treatments were used for the study of the short-term effects, and TS and CO mice of both sexes gestated by 40 dams under the three treatment conditions were used for the long-term effects study. All the experimental analyses were performed on the progeny of the pregnant-treated TS mice.

The offspring of these females were assigned to the one of the six experimental groups depending on their karyotype and the prenatal treatment that they received: CO pups that were treated prenatally with vehicle (CO-V), oleic acid (CO-OA), or linolenic acid (CO-LNA), and TS pups that prenatally received vehicle (TS-V), oleic acid (TS-OA), or linolenic acid (TS-LNA). For the long-term effects study, sixty-nine male and female TS and CO pups gestated by dams under the three treatments were assigned to the same experimental groups. Six to seven pups from each group were used to evaluate the short-term effects (i.e. neurogenesis, cellularity, and brain volume), and 10-13 juvenile mice prenatally treated with oleic acid, linolenic acid, or vehicle (n=10-13 per group; CO-V: n=10, TS-V: n=13, CO-OA: n=12, TS-OA: n=10, CO-LNA: n=12, TS-LNA n=12), were used to assess the long-term effects of the treatments (i.e. cognition in the Morris water maze (MWM), neurogenesis, cellularity, and pre- and post-synaptic markers).

Short-term effects of prenatal treatment

To evaluate the short-term effects of oleic and linolenic acid on cell proliferation, on PD2, all pups received an intraperitoneal injection of Bromodeoxyuridine (BrdU) (150 µm/g). Two hours later, they were weighed, euthanized by decapitation, and their brains were removed, weighed and fixed in paraformaldehyde (PFA) solution, transferred to 30% sucrose and frozen at -80° C. Coronal sections of 30 µm covering the whole hippocampus were cryosectioned, and stored at -20° C. From each animal 7 series containing 6-8 hippocampal sections were obtained to perform the histological

144 analyses: granule cell layer (GCL) volume, cell proliferation (BrdU) and granule cell
145 density (4′,6-Diamidino-2-phenylindole, DAPI staining).

146 *Long-term effects of prenatal treatments*

147 To evaluate the long-term effects of the different treatments on new neuron survival, on
148 PD15 all pups received an intraperitoneal injection of BrdU (150 µg/g). On PD21, all the
149 animals were weaned and subjected to the behavioral experiments (Morris Water
150 Maze, MWM) between PD30 and PD45. On PD45, the animals were euthanized by
151 decapitation and the brains of 6-7 animals per group were removed, fixed with PFA and
152 used for the following histological and immunohistochemical analyses: GCL volume,
153 cell proliferation (Ki67 immunohistochemistry), survival (BrdU immunohistochemistry),
154 pre- and post-synaptic density (Synaptophysin (SYN) and Postsynaptic Density Protein
155 95 (PSD95) immunohistochemistry). To this end, free-floating 50 µm coronal sections
156 covering the whole hippocampus were cryosectioned. Nine series, containing 6-8
157 hippocampal sections, were obtained from each animal.

158 **Nissl Staining**

159 Morphological analysis of GCL volume was performed on 1 of 7 series, for the short-
160 term studies, and on 1 of 9 series, for the long-term studies. Nissl staining was
161 performed as previously described (59). To calculate the GCL volume, each coronal
162 section of the brain was photographed and the images were analyzed using ImageJ
163 software (http://rsb.info.nih.gov/ij/). In all cases, the Cavaliery stereological method was
164 used.

165 **Cell Proliferation (Ki67 and BrdU immunofluorescence)**

166 To quantify the short-term effect of the treatments on cell proliferation in the GCL of
167 PD2 animals, BrdU immunohistochemistry was performed as previously described
168 (10). The primary antibody used was an AntiBrdU at 1:100 (Santa Cruz) in PBS

(Phosphate-Buffered Saline) -TX (Triton X) 0.1% supplemented with 1% goat serum. The total number of BrdU+ cells was counted in the selected sections with Zen 2.6 software in the GCL and calculated using the optical dissector method, as previously described (60). In each animal, the total number of positive cells per slice was divided by the volume of the GCL, to calculate the density of proliferating cells.

For the long-term analyses of cell proliferation, the protocol described by Stagni et al. (18) was followed. The primary antibodies used were a rabbit anti-Ki67 at 1:750 (Abcam, Cambridge, UK) diluted in Phosphate Buffer (PB) with 0.5% TX-100 and 0.1% BSA and an AntiBrdU at 1:100 (Santa Cruz). The total number of Ki67-positive cells or BrdU-positive cells in the selected sections was counted using an optical fluorescence microscope (Zeiss Axioskop 2 plus, 40x objective). The total number of positive cells was divided by the area of the Subgranular Zone (SGZ) (defined as the length of the SGZ multiplied by the thickness of the section) to determine the Ki67+ or BrdU+ cell density in the SGZ. The total number of Ki67+ cells and BrdU+ cells in the SGZ was calculated using the optical dissector method, as previously described (59, 60).

DAPI Staining

For the study of the short- and long-term effects on the number of mature cells, sections were counterstained with DAPI (Calbiochem, Billerica, MA, USA; 1:1000). In both the short and long-term analysis, cell counts were performed using a previously described physical dissector system coupled with confocal microscopy (61, 62).

SYN and PSD95 Immunofluorescence

For the long-term synaptic effects study, SYN and PSD95 immunohistochemistry were employed following the protocol previously described in Stagni et al. (18). The antibodies used were a mouse monoclonal anti-SYN (SY38) antibody (Millipore-Biomanufacturing and Life Science Research, Billerica, MA, USA) and a rabbit

polyclonal anti-PSD95 antibody (Abcam) both diluted to 1:1000. Fluorescent images were captured by a confocal microscope (Leica SP5), using a 63x 1.4 NA objective and an 8x zoom. For each marker, four sections per animal were used comprising the entire hippocampus, and one random area in the Molecular Layer (ML) of the Dentate Gyrus (DG), CA1, and CA3 per section was measured. Image analysis was performed using the NIH ImageJ software. For each marker, the number of individual puncta exhibiting SYN or PSD95 immunoreactivity was counted in a circle with an area of 325 μm^2 for each image in each hippocampal field.

Cognitive Analysis. Morris Water Maze (MWM)

Spatial learning and memory were evaluated using a modified version of the MWM (20). Sixteen consecutive daily sessions were performed: 12 acquisition sessions (platform submerged, in eight of these, the position of the platform changed daily, while in the other four it was kept constant), followed by a probe trial and 4 cued sessions (platform visible). The computerized tracking system Anymaze (Stoelting, Wood Dale, IL, USA) was used to analyze the trajectories of the animals and record escape latency, distance traveled, and swimming speed of each animal in each trial.

Statistics

Shapiro–Wilk tests were used to test the normality of the data sets. Because all of them were normally distributed, parametric tests were used. The water maze data from the acquisition sessions (sessions 1-12) were analyzed using two-way Analysis of Variance (ANOVA) with Repeated Measures (RM) ('session' x 'karyotype' x 'treatment' or 'trial' x 'karyotype' x 'treatment'). The rest of the data from the short-term and long-term studies was analyzed using two-way ('karyotype' x 'treatment') ANOVA or RM ANOVA ('quadrant'). The mean values of each experimental group were compared *post hoc* using Fisher's LSD (Least Significant Difference) *post-hoc* tests. The

differences between groups were considered to be statistically significant when *P*<0.05. All analyses were performed using IBM SPSS (Armonk, New York, USA) for Windows version 22.0.

RESULTS

Short-term Effects of Prenatal Treatment

1. Plasma Levels

After administration of the vehicle to TS females during pregnancy, their PD2 offspring showed a mean value of 9.3 µg/mL of oleic acid, and 2.7 µg/mL of linolenic acid in plasma. The PD2 pups whose TS mothers received oleic acid from ED10 to PD2 presented a mean value of 20.4 µg/mL of this fatty acid, while PD2 pups born from pregnant TS females that received linolenic acid during gestation showed a mean plasma level of 8.6 µg/mL of this acid.

2. Body and Brain Weight

At PD2, TS-V mice presented smaller body (p<0.05; **figure 1A**) and brain weights (*P*<0.01*;* **figure 1B**) than CO-V mice. Prenatal linolenic acid treatment increased the bodyweight of LNA-CO pups with respect to CO-V mice (*P*<0.001; **figure 1A**).

Prenatal administration of oleic acid increased the brain weight of TS-OA mice at PD2 with respect to TS-V pups (*P*<0.01). Linolenic acid administration increased the brain weight of mice of both karyotypes, as demonstrated by the significant increase observed in the TS-LNA group with respect to TS-V mice (*P*<0.001), and in CO-LNA when compared to CO-V pups (*P*<0.001; **figure 1B**).

3. Granular Cell Layer Volume

242 At PD2, the three groups of TS mice presented smaller volumes of the GCL than the
243 three groups of controls ('karyotype': *P*=0.012; **figures 1C and 1D**). Oleic acid
244 treatment increased the GCL volume in pups of both karyotypes (TS-OA vs. TS-V,
245 *P*<0.05, CO-OA vs. CO-V, *P*<0.05), while prenatal linolenic acid administration only
246 enhanced the GCL volume of CO mice with respect to the CO-V group (*P*<0.05;
247 **figures 1C and 1D**).

248 **4. BrdU Immunohistochemistry**

249 At PD2, TS-V mice presented a lower number of BrdU+ cells per slice (*P*<0.01, **figures**
250 **2A and 2B**), and a lower total number of cells than their CO-V littermates (*P*<0.01*;*
251 **figure 2C**). Both prenatal treatments increased the number of BrdU+ cells per slice in
252 TS pups (TS-OA vs. TS-V: *P*<0.001; TS-LNA vs. TS-V: *P*<0.01; **figure 2B**), and the
253 total number of BrdU+ cells (TS-OA vs. TS-V: *P*<0.01; TS-LNA vs. TS-V: *P*<0.01;
254 **figure 2C**). However, neither the density nor the total number of this population of cells
255 were significantly modified by any of the treatments in PD2 CO mice.

256 **5. Mature Granule Cell Count (DAPI Staining)**

257 TS-V mice presented a lower total number of DAPI+ cells than their CO-V littermates
258 (*P*<0.05; **figures 2D and 2F**), although the density of this population of cells did not
259 significantly differ between PD2 pups of both karyotypes (**figure 2E**). In TS mice, oleic
260 acid treatment increased the density (TS-OA vs. TS-V: *P*<0.05; **figure 2E**) and the total
261 number of DAPI+ cells (TS-OA vs. TS-V *P*<0.01; **figure 2F**), and both treatments
262 significantly increased the total number of this population of cells in CO animals (CO-
263 OA vs. CO-V *P*<0.05; CO-LNA vs. CO-V: *P*<0.05; **figure 2F**).

264 **Long-term Effects of Prenatal Treatment**

265 **1. Histology**

At PD45, TS-V mice presented a lower density (P<0.01; **Supplemental figure 1B**), and a lower total number of Ki67+ (P<0.01; **Supplemental figure 1C**) and BrdU+ (P<0.05; **Supplemental figure 1E**) cells than their CO-V littermates. Prenatal treatment with oleic or linolenic acid did not exert any long-term effect on the GCL volume, the density, or the total number of Ki67+ or of BrdU+ cells in TS mice, since neither TS-OA nor TS-LNA mice differed in any of these measurements from TS-V mice (**Supplemental figures 1A-1E**). However, at PD45, TS-LNA mice showed a higher density of mature DAPI+ cells than TS-V mice (P<0.05; **Supplemental figure 1F**).

At PD45, TS-V mice presented a smaller number of PSD95+ puncta than their CO-V littermates in all hippocampal areas analyzed (CA1: P<0.05; CA3: P<0.05; ML: P<0.01; **figures 3A and 3B**). Prenatal treatment with oleic acid produced a long-term enhancement of the number of PSD95+ puncta in TS mice (TS-OA vs. TS-V: CA1: P<0.05; CA3: P<0.01; ML: P<0.05); while treatment with linolenic acid increased the number of PSD95+ puncta in the ML of TS-LNA mice with respect to TS-V animals, although no changes were observed in the other hippocampal areas analyzed (CA1: P=0.65; CA3: P=0.14; ML: P<0.05).

Although TS-V mice presented a smaller number of SYN+ puncta than CO-V mice in CA1 (P<0.05), CA3 (P<0.01) and in the ML (P<0.01; **figures 3C and 3D**), none of the treatments modified the number of SYN+ puncta in TS or CO mice.

2. Cognition: Morris Water Maze

2.1. Reference learning and Memory

The six groups of mice reduced their latency to reach the platform when all sessions were taken into account (session 1-12: RM ANOVA ‘session’: P<0.001; **figure 4A**),

291 both in the sessions in which the platform position was changed daily (sessions 1-8:
292 *P*<0.001) and in those in which the platform position was kept constant (sessions 9-12:
293 *P*<0.001).

294 The reduction in latency between sessions significantly differed between animals of
295 both karyotypes ('session x karyotype': *P*<0.001) and of the three treatment conditions
296 ('session x treatment': *P*=0.001).

297 When each pair of learning curves was analyzed separately, TS-V mice presented a
298 deteriorated performance when compared to their CO-V littermates (*P*<0.001; **figure**
299 **4B).**

300 TS-OA (sessions 1-12: *P*=0.006; sessions 1-8: *P*=0.018; sessions: 9-12: *P*=0.026;
301 **figure 4C**), and TS-LNA mice showed lower latencies to reach the platform than TS-V
302 animals (sessions 1-12: *P*=0.007; sessions 1-8: *P*=0.020; sessions 9:12: *P*=0.059;
303 **figure 4E**).

304 In the case of the CO animals, prenatal administration of oleic acid did not modify their
305 performance in the MWM, since CO-OA mice did not differ in their latency to reach the
306 platform when compared with CO-V mice (sessions 1-12: *P*=0.11; sessions 1-8:
307 *P*=0.30; sessions 9-12: *P*=0.060; **figure 4D**). CO-LNA animals presented lower
308 latencies to reach the platform than the CO-V group only in the sessions in which the
309 platform position was kept constant (Sessions 1-12: *P*=0.23; sessions 1-8: *P*=0.37;
310 sessions 9-12: *P*=0.043; **figure 4F**).

311 **2.2. Working Memory**

312 When all the groups of mice were analyzed together, statistical analyses demonstrated
313 a marked reduction in their latency to reach the platform across trials (RM ANOVA
314 'trial': *P*<0.001; **figure 5A**). However, the reduction in these latencies differed between
315 the three groups of TS and the three groups of CO mice ('trial x karyotype': *P*<0.001),

316 and between TS and CO mice under the three treatment conditions ('trial x treatment':
317 *P*=0.037).

318 When each pair of learning curves was analyzed separately, it was observed that TS-V
319 animals showed a deteriorated working memory, since they did not reduce their latency
320 to reach the platform throughout the trials (*P*=0.82; **figure 5B**), and their latency to
321 reach the platform was higher than that of CO-V mice (*P*=0.001, **figure 5B**). However,
322 TS-OA (RM ANOVA 'trial': *P*=0.012, **figure 5C**) and TS-LNA mice (*P*=0.052; **figure 5E**)
323 reduced their latency to reach the platform between trials, indicative of their ability to
324 learn the platform position across trials.

325 In euploid mice, both groups of mice, CO-OA ('trial': *P*<0.001; **figure 5D**) and CO-LNA
326 (*P*<0.001; **figure 5F**) reduced their latency to reach the platform between trials.

327 **2.3. Cued Sessions**

328 TS and CO mice under the different treatments did not differ in their latency to reach
329 the platform during the cued sessions when the platform was visible ('karyotype':
330 *P*=0.14, 'treatment: *P*=0.30; karyotype x treatment': *P*=0.038; data not shown).

331 **2.4. Spatial Memory**

332 During the probe trial, TS-V mice crossed fewer times over the place where the
333 platform was placed during the training sessions (*P*<0.05; **figure 6A**) and entered the
334 trained quadrant fewer times (*P*<0.05; **figure 6B**) than their CO-V littermates.

335 Prenatal treatment with oleic acid or linolenic acid increased the number of crossings
336 that TS mice performed over the platform position with respect to vehicle-treated
337 trisomic animals (TS-OA vs. TS-V: *P*=0.05; TS-LNA vs. TS-V: *P*<0.05; **figure 6A**), but
338 did not exert any significant effect on the number of entries that TS or CO mice made
339 into the trained quadrant (**figure 6B**).

340 TS-V mice did not show a preference for any of the quadrants (*P*=0.54; **figures 6C** and
341 **6E**). However, prenatal oleic acid or linolenic acid treatment improved the spatial
342 memory of TS mice, since TS-OA and TS-LNA animals spent more time in the trained
343 quadrant than in the rest of the quadrants (TS-OA: *P*<0.001; TS-LNA: *P*<0.001; **figures**
344 **6C** and **6E**). The three groups of CO mice spent a higher percentage of time in the
345 trained quadrant than in the rest of the quadrants (CO-OA: *P*<0.001; CO-LNA:
346 *P*<0.001; CO-V: *P*<0.001; **figures 6D** and **6F**).

347

DISCUSSION

In this study, prenatal administration of oleic acid and linolenic acid rescued several neuromorphological alterations found in newborn TS mice. In particular, these treatments increased their brain weight, the volume of their GCL, and the number of proliferating and mature granule cells in their hippocampi. When the effects of these treatments were analyzed six weeks after the discontinuation of the treatment, no significant effects were found in the volume of the GCL, the number and density of Ki67+ or BrdU+ cells or the number of SYN+ puncta found in the hippocampus of TS mice. However, both treatments increased the number of PSD95+ puncta. When the long-term functional effects of both prenatal treatments were analyzed, TS mice that received oleic acid or linolenic acid showed improved reference, working, and spatial memory in the Morris water maze.

Both oleic acid and linolenic acid have been demonstrated to cross the placental and blood-brain barrier (35, 47, 63). In this study, the pups of the dams treated with these acids presented an increased level of both acids in plasma. Thus, oleic acid and linolenic acid were adequately transferred to the pups, incorporated into the bloodstream and distributed throughout the organism.

Consistent with the altered prenatal neurodevelopment of TS mice and DS individuals (2, 3, 10, 11, 14, 21, 64), we found that TS mice presented reduced brain weight, GCL volume, cell proliferation and density of mature cells. Both lipid composition and fatty acid metabolism are essential for proper neurodevelopment. DS individuals present lower levels of monounsaturated fatty acids, especially of oleic acid, in their brains (52, 65). Thus, these deficiencies could play an essential role in the neurodevelopmental alterations and/or in the functional anomalies found in DS. However, there is increasing evidence that brain development is not only affected by the total levels of fatty acids, but also by the ratio and the relationship between these acids. In DS, corn oil that is a

source of linoleic acid, a precursor of PUFAs of the omega-6 series, which produces arachidonic acid (ARA), improves brain development (66), supporting the idea of the essential role of omega-6 and ARA in neurodevelopment. Furthermore, DS brains present an increased ratio of PUFAs of the omega-3 series with respect to those of the omega-6 series (65). These authors proposed that this ratio was modified due to the decrease in ARA brains and that this effect plays a role in the alterations in neurodevelopment found in in DS. Therefore, in the present study it is possible that adding omega-3 PUFAS to the diet induced a reduction in ARA levels, thereby increasing the total amount of omega-3 fatty acids and enhancing the omega-3: omega-6 ratio. This effect could be partially responsible for the benefits found after its administration.

In the present study, prenatal administration of oleic and linolenic acid increased cell proliferation in the hippocampi of TS pups and reduced the hypocellularity in this structure. There is evidence of the pro-neurogenic effect of both acids in different conditions (25, 32-34, 47, 48, 66). Prenatal administration of compounds that increase neurogenesis, such as the serotonin reuptake inhibitor Fluoxetine, normalizes the neuroanatomical anomalies in TS mice (11). Thus, the increase in granule cell density induced by these compounds could be due to the enhanced cell proliferation, differentiation, and/or survival.

During neurodevelopment, the primary role of oleic acid in neurogenesis is to act as a neurotrophic factor (26, 27). During this period, brain development is regulated by the Alpha-Fetoprotein (AFP)/albumin ratio that modulates the neurotrophic effects of oleic acid (67). Thus, the balance between these two signals seems to be essential to induce neurogenesis in embryonic development, while its imbalance contributes to the onset of several alterations during neurodevelopment. DS individuals present a reduction in the serum levels of AFP and albumin (68, 69) that correlates with a lower

concentration of oleic acid in the brain (65). Thus, it is possible, that after the administration of oleic acid during the prenatal stages, adequate levels of this fatty acid were reached, thereby correcting the alterations in hippocampal neurogenesis in TS mice. However, after discontinuation of the treatment, these levels would likely have returned to their original altered states, and this might be one of the reasons why the beneficial effects of oleic and linolenic acid on neurogenesis in TS mice are not seen one month later.

Regarding linolenic acid, it has been demonstrated that during gestation and in the first stages of postnatal life an adequate intake of omega-3 fatty acids, such as linolenic acid, is necessary to allow for the correct development of the brain and neurogenesis (32). Most of the beneficial effects of omega-3 fatty acids are due to their conversion into DHA. Numerous studies have demonstrated that the administration of a diet rich in linolenic acid promotes hippocampal neurogenesis (33, 34, 70). In contrast, DHA deficiency during gestation, due to the reduced intake of linolenic acid, alters brain morphology, including the cortical and hippocampal areas, as well as hippocampal neurogenesis (32). These authors suggest that the inhibition of neurogenesis which occurs after linolenic acid restriction during gestation could be due to a delay in the cell cycle or the onset of neurogenesis. In TS mice, alterations in neurogenesis during prenatal stages are also due to a slowing-down of the cell cycle (1, 10, 14). Thus, the administration of linolenic acid to pregnant TS females could accelerate the speed and/or reduce alterations in the cell cycle, increasing the number of neuronal precursors in TS pups.

Oleic and linolenic acid treatment also enhanced the number of mature granular cells, possibly due to the well-known effect of both acids on neuronal differentiation and survival (30, 70-72). This increased cellularity is likely to be responsible for the increase in the GCL volume and brain weight found in TS mice after prenatal oleic and linolenic

acid treatment. Linolenic acid treatment increases hippocampal volume (47, 72). Conversely, restriction of linolenic acid in the diet during gestation alters the normal development of the brain (32). Because low levels of fatty acids are found in DS brains (52, 65), their exogenous administration to pregnant TS mice could positively impact prenatal brain development, restoring the size of the hippocampal structures in their progeny.

However, most of the beneficial effects of the prenatal administration of oleic and linolenic acid on the neuromorphological alterations found in TS mice were not maintained six weeks after the discontinuation of the treatment. These results indicate that the presence of oleic acid and linolenic acid may be necessary to induce their proliferative and pro-survival effects in TS mice.

Administration of fatty acids during adulthood also reduces the neuropathology found in TS mice. Giacomini et al. (66) showed that treatment with corn oil, which contains both oleic and linoleic acid, rescued brain weight, neurogenesis, dendritogenesis and cognition in adult TS mice. In addition, using cultures of neural progenitor cells (NPCs) obtained from DS individuals, they demonstrated that both linolenic and oleic acid can increase the proliferation rate of NPCs (66).

One of the most relevant results of this study is the finding that prenatal administration of oleic and linolenic acid produced long-term enhancement of the cognitive abilities of TS mice in the MWM. Those animals that received this treatment during gestation demonstrated better reference, working, and spatial memory than the ones that received the vehicle, as indicated by their reduced latency to reach the platform across sessions (reference memory), and across trials in each session (working memory). Finally, both treatments also increased the memory of the platform position in TS mice, since these animals spent more time searching for the platform position in the trained quadrant and crossed over the platform position more times than the vehicle-treated

453 TS mice. These results agree with the literature that reports the pro-cognitive effects of
454 oleic and linolenic acid and their derivatives. DHA supplementation improves cognition
455 and spatial memory in normal rodents, in murine models of Alzheimer's disease (AD),
456 (49, 50) and in the pups of rats supplemented with linolenic acid during gestation (51).
457 Moreover, administration of corn oil to adult TS mice also induces pro-cognitive effects
458 (66), and treatment with a diet supplemented with fish oil that contained linolenic acid
459 rescued cognitive deficits in mice that overexpressed the *RCAN-1* gene which is
460 triplicated in DS and in TS mice (73).

461 The pro-cognitive effects found in this study cannot be attributed to changes in
462 neurogenesis. However, changes in the characteristics of the synaptic connections
463 might be partially responsible for these beneficial effects in the cognitive abilities of TS
464 mice.

465 Although the total number of synapses was unchanged after both treatments, as
466 demonstrated by the similar number of SYN+ puncta found in TS mice under all
467 treatments, oleic acid administration increased the number of PSD95+ puncta, a
468 postsynaptic marker of excitatory synapses (74, 75), in all hippocampal areas
469 analyzed. Linolenic acid treatment only increased PSD95+ in the ML of the
470 hippocampus. Since the number of synapses was similar in all groups, these results
471 suggest that the altered inhibitory-excitatory balance of TS mice could have been
472 partially corrected. The over-inhibition, due to enhanced GABAergic transmission and
473 reduced glutamatergic transmission, characteristic of TS mice, has been proposed to
474 play an essential role in the cognitive difficulties of TS mice (76). Among the
475 mechanisms proposed as being responsible for the beneficial effects of oleic and
476 linolenic is the increase in glutamatergic transmission, through the increased
477 expression of vesicular glutamate transporters 1 and 2 (VGluT1 and VGluT2) (33, 71,
478 72). Oleic acid promotes the synthesis of the post-synaptic protein PSD95 (30), which

is implicated in Long-Term Potentiation (LTP) generation (77). Thus, an increase in excitatory transmission could be partially responsible for the long-term benefits exerted by oleic acid and linolenic acid in the reference memory, working memory, and spatial memory of TS mice found in this study.

In conclusion, prenatal administration of oleic and linolenic acid restored several neuromorphological alterations in the TS65Dn mouse model of DS. In addition, they produced long-term enhancement of the cognitive abilities of these animals, possibly by normalizing the excitatory-inhibitory synaptic balance. These results provide evidence for the potential therapeutic effect of oleic and linolenic acid in DS. Given that both are natural substances present in the human diet, they could be administered prenatally and thereby exert stronger effects on the neurodevelopment of the DS population.

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FIGURE LEGENDS

Figure 1. Body (A) and Brain (B) weights, and GCL Volume (C) of PD2 TS and CO mice prenatally treated with oleic acid, linolenic acid, or vehicle. Representative microscopy sections of Nissl staining in the hippocampus of the six groups of mice (D). Values in A, B and C are means ± SEMs, n=6-7 per group. Labeled bars without a common letter differ by $P < 0.05$, by Fisher's LSD *post-hoc* tests Scale bar in D: 200 µm.

Figure 2. Representative confocal images of BrdU+ immunohistochemistry (A) and by DAPI staining (D), number of BrdU+ cells per slice (B), the total number (C) of BrdU+ cells, and of the density (E) and total number (F) of DAPI+ cells in the hippocampus of TS and CO pups prenatally treated with oleic acid, linolenic acid, or vehicle. Scale bar in A: 100 µm, Scale bar in D: 5 µm. Values in B, C, E and F are means ± SEMs, n=6-7 per group. Labeled bars without a common letter differ by $P < 0.05$, by Fisher's LSD *post-hoc* tests.

Figure 3. Representative confocal images PSD95 (A), and SYN (C) immunochemistry in the hippocampus, number of PSD95+ (B), and SYN+ (D) puncta in the CA1, CA3 areas and ML of the hippocampus of 45-days-old TS and CO mice that received oleic acid, linolenic acid, or vehicle prenatally. Scale bars in A and C: 5 µm. Values in B and D are means ± SEMs, n=6-7 per group. Labeled bars without a common letter differ by $P < 0.05$, by Fisher's LSD *post-hoc* tests.

Figure 4. Latency to reach the platform during the twelve acquisition sessions in the MWM exhibited between PD30 and PD45 by all groups of mice (A), by TS-V and CO-V mice (B), by TS-OA and TS-V mice (C), by CO-OA and CO-V mice (D), by TS-LNA and TS-V mice (E), and by CO-LNA and CO-V mice (F). Values are means ± SEMs, n=10-13 per group. *: p<0.05, **: p<0.01, ***: p<0.001 vs. CO-V (in B,

D and F) or vs. TS-V (in C and E), Fisher's LSD *post-hoc* tests. On the right side of each figure, the *P*-value of the difference between both learning curves across the twelve sessions (RM ANOVAs) is shown. On top of each figure, the *P* values of the differences between the learning curves of the different groups of mice during the first 8 and the last 4 sessions are shown.

Figure 5. Latency to reach the platform during each trial of the first eight acquisition sessions exhibited between PD30 and PD45 by all animals (A), by TS-V and CO-V mice (B), by TS-OA and TS-V mice (C), by CO-OA and CO-V mice (D), by TS-LNA and TS-V mice (E), and by CO-LNA and CO-V mice (F), in the MWM. Values are means ± SEMs, n=10-13 per group. *: p<0.05, **: p<0.01, ***: p<0.001, vs. CO-V (in B, D and F) or vs. TS-V (in C and E) Fisher's LSD *post-hoc* tests. On the right side of each figure, the *P*-value of the main effects for 'karyotype' (figure B), or 'treatment': (figures C-F) after RM ANOVAs, are shown. The dotted lines and the *P*-values beside them represent the significance of the change in latency across the trials (RM ANOVA 'trial' of each learning curve).

Figure 6. Number of crossings over the platform position (A) and Number of entries in the trained quadrant (B) performed at PD45 in the probe trial by TS and CO mice prenatally treated with oleic acid, linolenic acid, or vehicle; Percentage of time spent in each quadrant during the probe trial by TS-OA, TS-LNA, and TS-V (C), and CO-OA, CO-LNA, and CO-V animals (D), and Percentage of time spent in the trained quadrant vs. the rest of the quadrants by TS-OA, TS-LNA, and TS-V (E), and CO-OA, CO-LNA, and CO-V (F) mice. Values are means ± SEMs, n=10-13 per group. Labeled bars without a common letter differ by $P < 0.05$, by Fisher's LSD *post-hoc* tests. ***: p<0.001 trained quadrant vs. the rest of the quadrants, Fisher's LSD *post-hoc* tests. The dotted lines in figures C-F represent the chance level, i.e. a probability equal to 25% of the time.

Figure 1

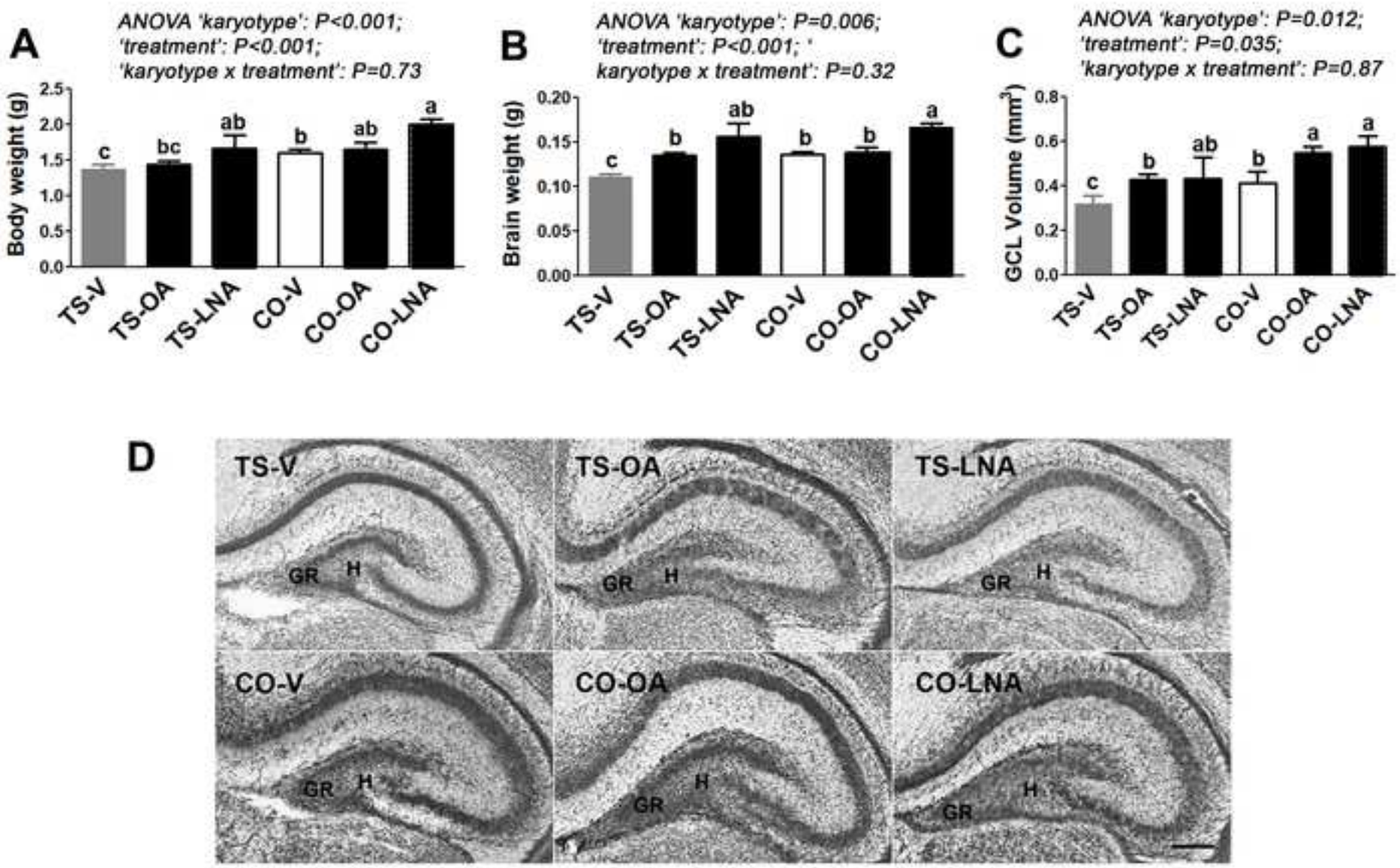


Figure 2

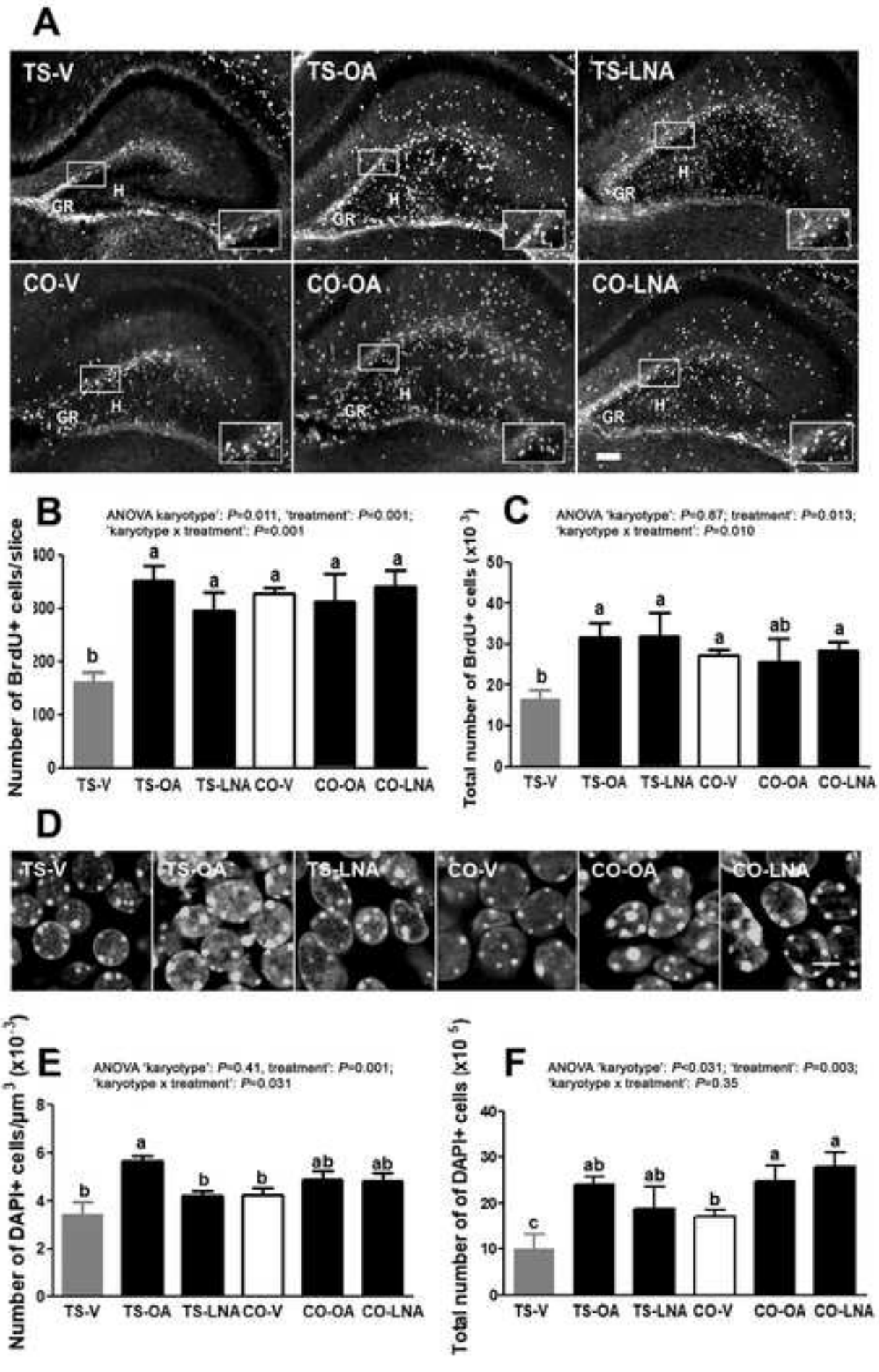


Figure 3

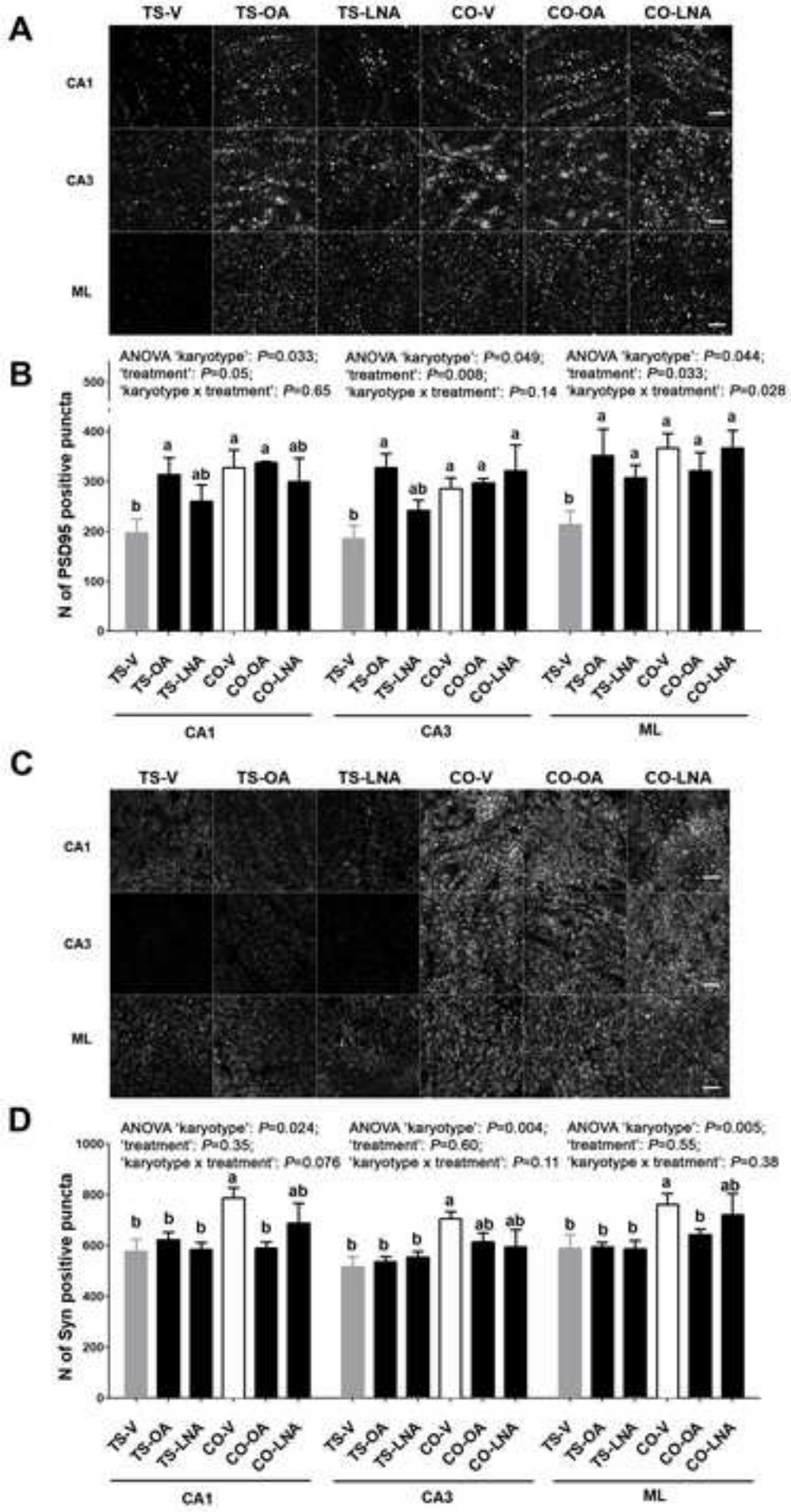
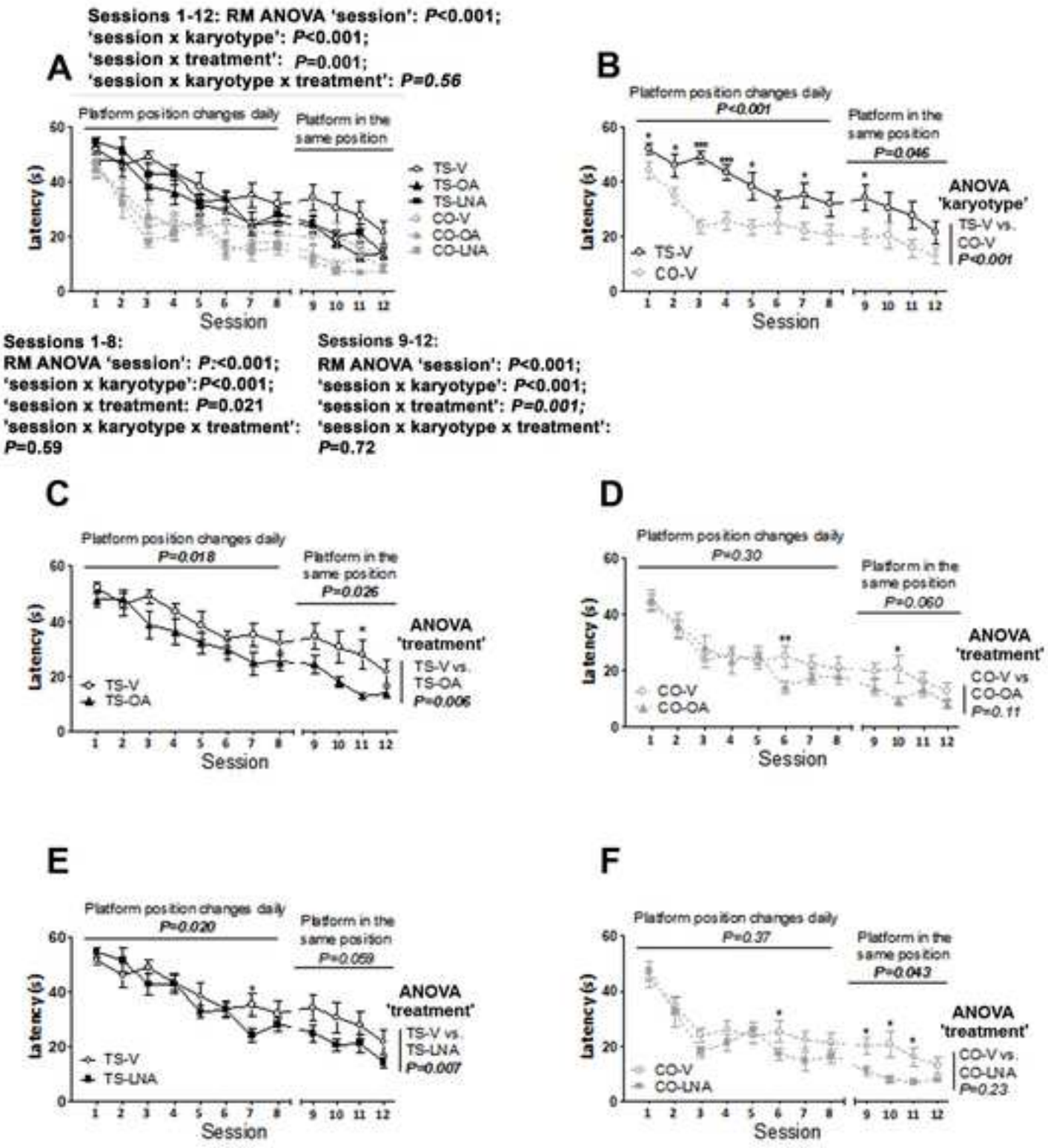


Figure 4



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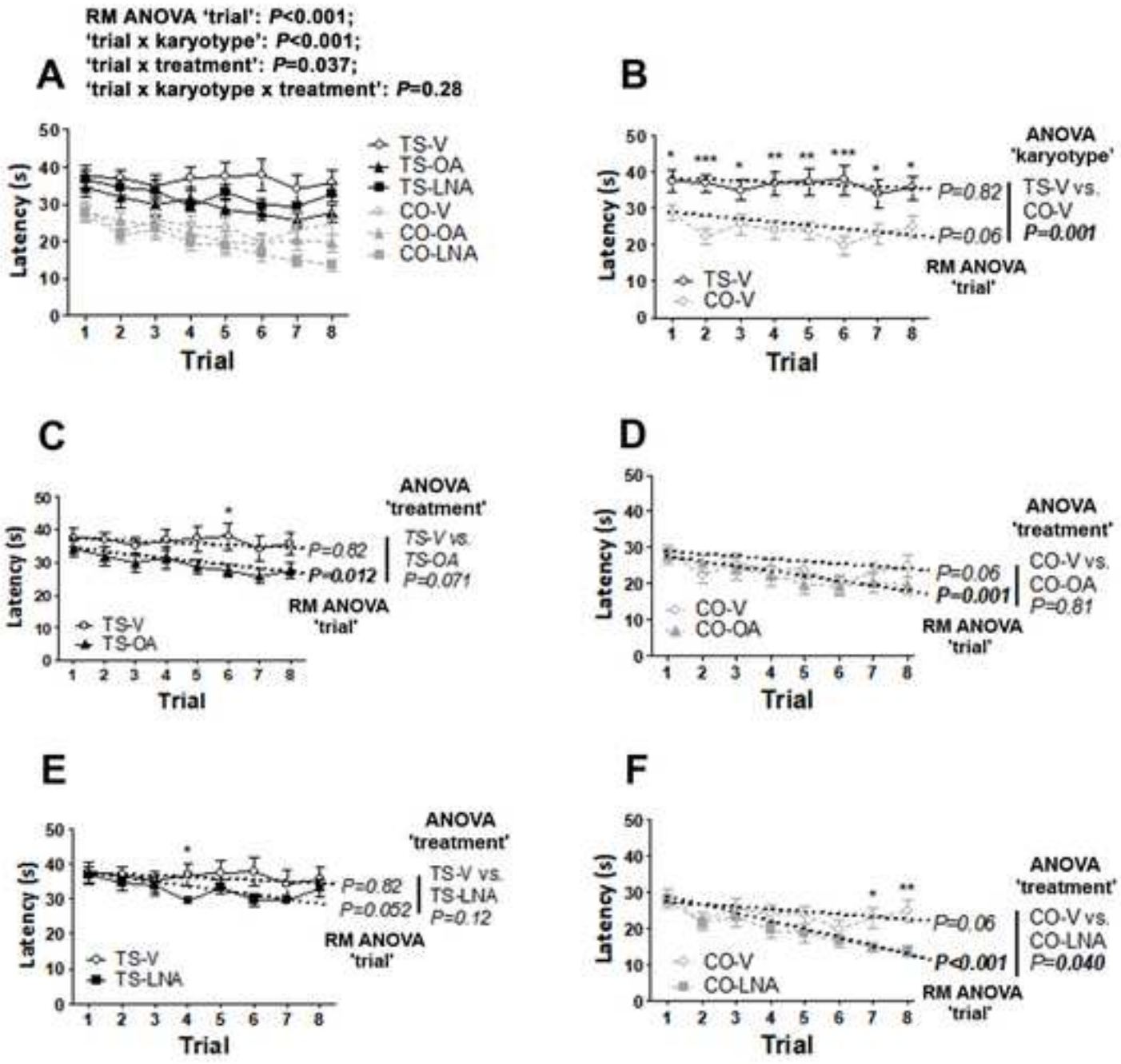
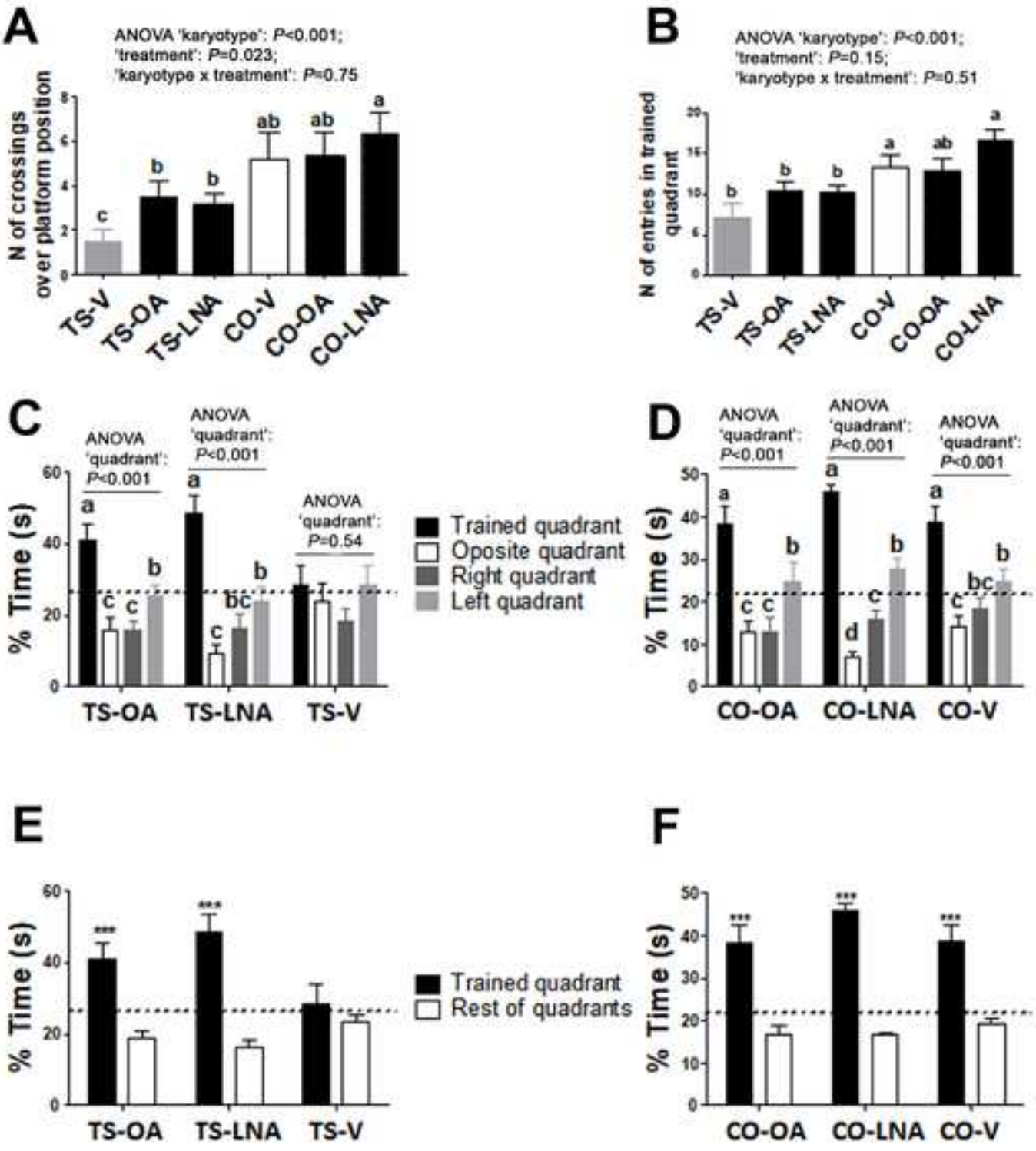


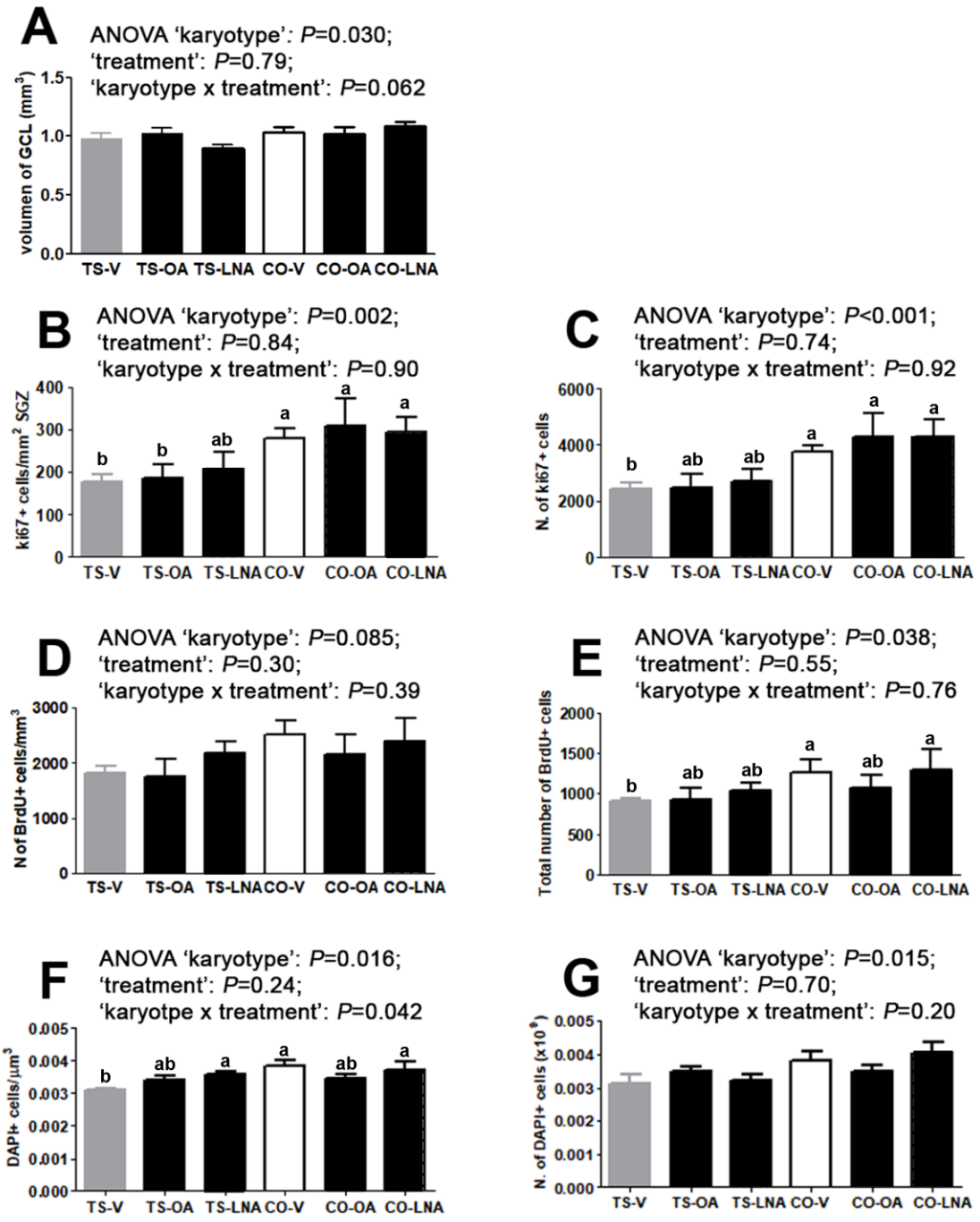
Figure 6

Click here to access/download;Figure;Figure 6.tif



"Prenatal administration of oleic or linolenic acid reduces neuromorpholotical and cognitive alterations in Ts65Dn Down syndrome mice". García-Cerro et al.

Online Supplementary Material



Supplementary figure 1. GCL volume (A); density (B) and total number (C) of Ki67+ cells; density (D) and total number (E) of BrdU+ cells; density (F) and total number of DAPI-stained cells in the hippocampus of 45 days-old TS and CO animals that received oleic acid, linolenic acid, or vehicle during gestation. Values are means ± S.E.M.s, n=6-7. Labeled bars without a common letter differ by *P*<0.05. Fisher's LSD *post-hoc* tests.